

Selected Paper

Speciation and Excretion Patterns of Arsenic Metabolites in Human Urine after Ingestion of Edible Seaweed, *Hizikia fusiforme*

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Chemical speciation of arsenic in human urine was performed by HPLC separation with ICP-MS detection in order to investigate the urinary excretion patterns of arsenic metabolites after one-time ingestion of an edible seaweed, *hijiki* (*Hizikia fusiforme*). A Japanese male volunteer ingested one serving of ca. 15 g (dry weight) of *hijiki* (containing ca. 0.9 mg of arsenic), and urine samples were collected at 3–5 h intervals for the following 3 days. As a result, different urinary excretion patterns were observed between inorganic and methylated arsenicals. Toxic inorganic As(V) and As(III) were detected in the urine 3 h after ingestion. The highest concentrations of As(V) and As(III) were observed at 6 and 15 h after ingestion, respectively. In contrast, methylated arsenic species in the urine, such as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and arsenobetaine (AB), began to increase 10 h after ingestion and provided the highest concentrations at 21 h after ingestion. These results indicate that a large amount of As(V) from *hijiki* is excreted rapidly in urine and some part of it is reduced to As(III) in the human body. As(III) may then be converted to non-toxic methylated arsenic species and excreted in urine later than inorganic ones. It can be concluded from these experimental results that detoxification of arsenic occurs in the human body through the rapid urinary excretion of toxic arsenicals or by conversion to organic methylated ones after ingestion of *hijiki*.

Chemical speciation analysis has been receiving great attention in environmental and biological sciences because the toxicities and bioavailabilities of various elements depend significantly on their chemical forms.[1–4] Since it is known that inorganic As(V) and As(III) compounds are highly toxic, arsenic speciation in various environmental and biological samples, such as water, soil, marine organisms, blood serum, urine, and so forth have been extensively studied so far.[5–10] It is also known that arsenic concentration levels are considerably high ($\mu g\,g^{-1}$ level) in marine organisms, many of which are food products for humans, although its level in seawater is not so high (ca. 0.0012 $\mu g\,mL^{-1}$).[1] In addition, major arsenic species in fish and seaweed have been elucidated as arsenobetaine and arsenosugars, respectively. In our daily life, thus, ingestion of arsenic-rich seafoods results in a significant intake of arsenic, making the elucidation of arsenic metabolism in the human body imperative.

Urinary excretion is the major pathway for the elimination of arsenic, and thus, the analysis of arsenic in urine samples is effective in elucidating arsenic metabolism in the human body after the ingestion of seafood. So far, many studies on arsenic speciation in urine after the ingestion of seafood have been reported.[11–17] Le et al. examined human urinary arsenic excretion patterns after the ingestion of seaweed (*nori* and kelp), crab, and shrimp.[16] They reported the rapid urinary excretion of arsenic after the ingestion of crab and shrimp, and a longer retention of arsenic in the human body when seaweeds were ingested.

On the 28th of July, 2004, the Food Standards Agency in UK warned not to eat a type of seaweed called *hijiki* (*Hizikia fusiforme*) because it contained a large concentration of arsenic (ca. 20–100 μg of arsenic per gram dry weight) compared to other seaweeds, especially inorganic arsenic species, which are considered human carcinogens. Two days after the warning, the Ministry of Health, Labor and Welfare in Japan reported that arsenic intake from *hijiki* in daily life caused no problems; average daily consumption of *hijiki* by Japanese is ca. 0.9 g, not enough to exceed the PTWI (provisional tolerable weekly intake) for inorganic arsenic (15 μg per kg body weight per week) reported by WHO. In fact, health effects due to arsenic poisoning after ingestion of *hijiki* have never been reported in the world. However, little is known about the arsenic metabolites in the human body after eating *hijiki*, although urinary excretion of arsenic species after ingestion of water extracts of *hijiki* was reported by Yamauchi et al.[14] Therefore, in the present research, speciation of arsenic in human urine was carried out by HPLC/ICP-MS to elucidate urinary excretion patterns of arsenic metabolites after a one-time ingestion of *hijiki*.

Experimental

Instrumentation. The chromatographic system consisted of a pump (model LC-10Ai, Shimadzu, Kyoto, Japan), a sample injector (model 7725, Rheodyne, Cotati, CA, USA) with a 20 μL loop, an ODS column (L-column, 4.6 mm i.d. × 250 mm long; Chemicals Evaluation and Research Institute, Tokyo, Japan), and an ICP-MS instrument (model HP 4500, Agilent Technologies, Yokogawa Analytical Systems, Tokyo, Japan). The conditions of ion-pair chromatographic separation were optimized according to the procedure by Shibata et al.[18] The mobile phase used in the present experiment was 4 mM malonic acid in 0.5% methanol with ion-pairing reagents of 4 mM tetramethylammonium hydrox-

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Table 1. Operating Conditions of the HPLC/ICP-MS Hyphenated System

HPLC	
Column	L-Column (ODS column)
Mobile phase	4 mM malonic acid, 4 mM tetramethylammonium hydroxide, and 10 mM sodium *n*-butanesulfonate in water/methanol (99.5:0.5, v/v), pH 3.0
Flow rate	0.75 mL min^{-1}
Sample injection volume	20 μL
ICP-MS	model HP 4500 (Agilent Technologies)
Plasma conditions	
Rf frequency	27.12 MHz
Rf power	1.3 kW
Gas flow rate	
Coolant gas	Ar 15 L min^{-1}
Auxiliary gas	Ar 1.0 L min^{-1}
Carrier gas	Ar 1.0 L min^{-1}
Sampling conditions	
Sampling depth	6 mm from work coil
Sample uptake rate	0.1 mL min^{-1}
Data acquisition	
Data point	1 point/peak
Dwell time	100 ms/point
Measurement time	800 s

ide and 10 mM sodium *n*-butanesulfonate, where pH was adjusted to 3.0 by adding nitric acid. The operating conditions of HPLC and ICP-MS are summarized in Table 1.

Urinary sodium was determined by ICP-AES (model Plasma AtomComp Mk II, Jarrell-Ash, Franklin, MA, USA) in order to correct variations in urine volume.

Chemicals and Samples. Standard compounds of arsenate (As(V)), arsenite (As(III)), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), arsenobetaine (AB), arsenocholine (AC), and tetramethylarsonium ion (TeMA) were purchased from Wako Pure Chemicals (Osaka, Japan). Other reagents used in the present experiment were of analytical reagent grade. Malonic acid, sodium *n*-butanesulfonate, and methanol were obtained from Wako Pure Chemicals, tetramethylammonium hydroxide (TMAH) from Tama Chemicals (Tokyo, Japan), and nitric acid from Kanto Chemicals (Tokyo, Japan). Pure water used throughout the present experiment was prepared by a Milli-Q water purification system (model Element A-10, Nihon Millipore Kogyo, Tokyo, Japan).

Extraction Procedure of Arsenic Species from *Hijiki*. The edible seaweed, *hijiki*, was purchased from a local food store. Approximately 0.2 g (dry weight) of *hijiki* was placed in a centrifugation tube. After adding 5 mL of pure water to the tube, the sample was kept for 30 min at room temperature. The supernatant was collected as the water-extracted sample solution. The residue was then homogenized and 5 mL of methanol/water (50:50, v/v) was added. The sample was sonicated for 30 min and centrifuged at 3000 rpm for 5 min. The supernatant was evaporated to remove methanol, and the residue was dissolved with 5 mL of the mobile phase solution. Both water- and methanol-extracted sample solutions were filtered with a membrane filter (DISMIC-25HP, pore size 0.45 μm, ADVANTEC, Tokyo, Japan), and subjected to the HPLC/ICP-MS measurement.

Collection of the Urine Samples after Ingestion of *Hijiki*. A Japanese male volunteer ingested ca. 15 g (dry weight) of *hijiki*, together with daily low-arsenic foods in one meal (initial time; 0 h). *Hijiki* was cooked after being kept in water for ca. 1 h. After ingestion of *hijiki*, urine samples were collected at 3–5 h intervals for the following 3 days. During the experimental period, a low-arsenic diet was ingested. A urine sample before ingestion was also collected to determine the background arsenic concentration. All urine samples were filtered with a membrane filter and stored at 4 °C until analysis.

Results and Discussion

Analysis of Standard Arsenic Compounds by HPLC/ICP-MS. Prior to the identification and determination of arsenic species in urine, standard arsenic compounds were analyzed by the present HPLC/ICP-MS system. Figure 1 shows the HPLC chromatograms of arsenic standards under two different mobile phase conditions. It can be seen in Fig. 1a that eight arsenic compounds of arsenate (11.6 ng mL^{-1} As), arsenite (15.4 ng mL^{-1} As), MMA (10.4 ng mL^{-1} As), DMA (11.6 ng mL^{-1} As), AB (11.8 ng mL^{-1} As), TMAO (9.9 ng mL^{-1} As), TeMA (9.5 ng mL^{-1} As), and AC (9.9 ng mL^{-1} As) were separated from each other within 800 s when the mobile phase including 0.05% of methanol was used, which was the same condition reported previously by Shibata et al.[18] As can be seen in Fig. 1b, the resolution of biochemically important DMA and AB was improved by raising the methanol concentration from 0.05 to 0.5%, although the peaks of TMAO and TeMA overlapped. As described later, DMA and AB were detected in all urine samples as well as in the extracts from *hijiki*, while TMAO and TeMA were not. Thus, the mobile phase including 0.5% of methanol was used in the following experiments.

Speciation of Arsenic in the Extracts from *Hijiki*. Figure 2 shows the chromatograms of arsenic compounds in the extracts from *hijiki* with water (a) and methanol (b). As can be seen in Fig. 2a, a large amount of As(V) was found

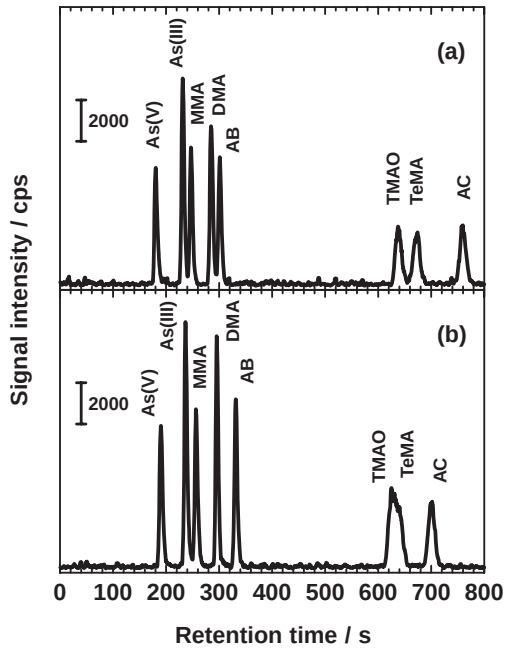

Fig. 1. Chromatograms of standard arsenic compounds with ICP-MS detection at m/z 75. The methanol concentration in the mobile phase: (a) 0.05% and (b) 0.5%. Column: L-Column (ODS column), Mobile phase: methanol aqueous solutions (pH 3.0) containing 4 mM malonic acid, 4 mM tetramethylammonium hydroxide, and 10 mM sodium *n*-butanesulfonate, Flow rate: 0.75 mL min^{-1}, Sample injection volume: 20 μL.

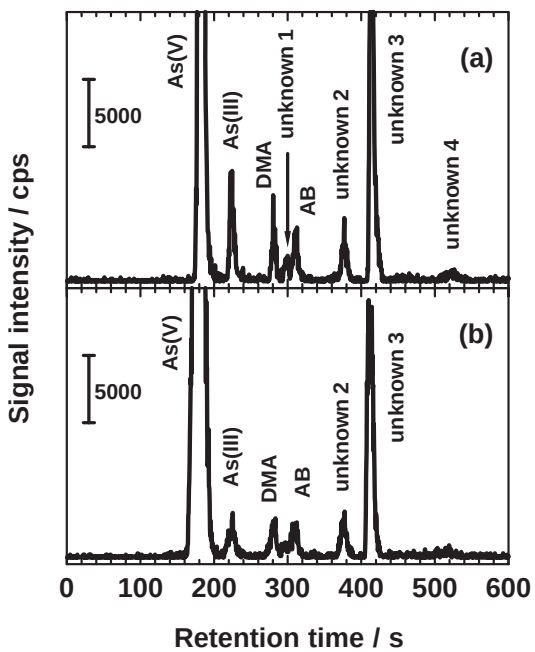

Fig. 2. Chromatograms of arsenic species in the extracts from *hijiki* with (a) water and (b) 50% methanol. Chromatographic conditions were the same as in Fig. 1.

in the water extract, and As(III), DMA, and AB could also be observed. In addition, four arsenic compounds were detected at 300, 380, 410, and 520 s, which have not been identified yet,

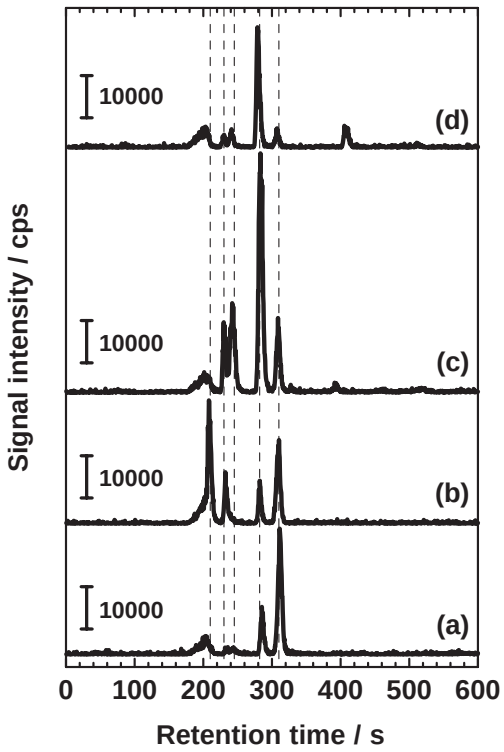

Fig. 3. Chromatograms of arsenic metabolites in human urine. (a) before, (b) 3 h after, (c) 21 h after, and (d) 50 h after ingestion of *hijiki*. Chromatographic conditions were the same as in Fig. 1.

and are indicated as unknown species 1, 2, 3, and 4, respectively, in the chromatogram. These extracted arsenicals were not taken into the human body, because *hijiki* was rinsed with an excess of water before cooking. In order to investigate arsenic species practically ingested into the body, the cooked *hijiki* was homogenized and extracted with 50% methanol. The result for the methanol-extracted sample is shown in Fig. 2b. As can be seen in Fig. 2b, large amounts of arsenicals were also found in the methanol-extracted sample, although unknown species 1 and 4 were not observed. These results indicate that various arsenic species, especially toxic inorganic As(V), still remained in *hijiki* even after the extraction with water. The remaining arsenicals accounted for 70–80% of the total arsenic in *hijiki*, which was calculated from the arsenic contents in *hijiki* both before and after rinsing with water.

Speciation of Arsenic in Human Urine after Ingestion of *Hijiki*. Figure 3 shows chromatographic separations of arsenic metabolites in urine samples successively collected from a healthy volunteer. The total arsenic content in the cooked *hijiki* was ca. 900 μg, which was determined by ICP-MS after acid digestion. As can be seen in Fig. 3, some peaks were detected at 210, 230, 245, 280, and 310 s, corresponding to As(V), As(III), MMA, DMA, and AB, respectively. TMAO, TeMA, and AC were not found in all urine samples. A broad peak was observed around 200 s in the present experiment, which was attributed to a polyatomic interference of $^{40}Ar^{35}Cl$ in the ICP-MS measurement, because a large amount of Cl (ca. 3000 μg mL^{-1}) was contained in the urine samples.

As can be seen in Fig. 3a, the contents of As(V) and As(III)

Table 2. The Observed and Na-Normalized Concentrations of Arsenic in Human Urine before and after Ingestion of *Hijiki*

Time	Observed concentration[a)]/ng mL^{-1}					Na-normalized concentration[a)]/$\times 10^{-7}$				
	As(V)	As(III)	MMA	DMA	AB	As(V)	As(III)	MMA	DMA	AB
before	0	1.8	1.5	11.9	41.8	0	6.3	5.5	42.4	149
3	**34.0**	11.7	0.7	9.7	26.9	89.2	30.6	1.8	25.3	70.6
6	18.9	13.9	2.9	8.8	7.6	**103**	75.7	15.7	48.0	41.5
15	3.4	**22.9**	20.5	43.8	13.0	14.2	**95.1**	84.9	182	54.0
18.5	0	12.8	19.4	45.3	10.7	0	65.2	98.9	231	54.5
21	0	18.2	**29.4**	**78.2**	22.2	0	79.3	**128**	**340**	96.6
24	0	10.8	18.6	56.6	25.6	0	40.4	69.3	211	95.5
27	0	9.8	16.0	58.8	21.4	0	22.2	36.4	134	48.5
36.5	0	5.2	8.5	40.5	10.2	0	20.0	32.4	155	39.2
41	0	7.1	11.0	67.0	18.3	0	22.1	34.1	209	57.0
44	0	4.6	6.4	41.6	10.2	0	9.8	13.6	88.9	21.7
48	0	3.5	5.2	34.3	6.6	0	6.2	9.3	61.3	11.9
50	0	3.2	4.5	33.3	5.4	0	5.8	8.1	60.0	9.7
52	0	3.3	5.1	38.6	6.3	0	5.6	8.6	65.3	10.7
54	0	6.8	8.9	76.1	10.1	0	13.7	17.8	153	20.2
59	0	4.4	6.6	50.4	**28.8**	0	19.7	29.3	224	**128**
62.5	0	4.2	5.4	41.8	21.3	0	10.9	14.1	109	55.8
65.5	0	2.8	2.9	21.8	10.0	0	6.8	7.2	53.8	24.6
68.5	0	3.6	4.4	35.3	13.0	0	8.9	10.7	86.9	31.9

a) The values shown in the bold letters indicate the highest concentrations of arsenicals observed in the urine samples after ingestion of *hijiki*.

were significantly smaller in the urine sample before ingestion. The observation of DMA and AB in the urine sample before ingestion might be due to the intake of seafood before the experiment. It can be seen in Fig. 3b that clear peaks for As(V) and As(III) are present at 3 h after ingestion. In the 21-h urine sample, as shown in Fig. 3c, the signal intensity for As(V) returned to the background level, while those for MMA and DMA increased substantially. The concentration of DMA was 7 times higher in the 21-h urine sample than in urine before ingestion. At 50 h after ingestion, as shown in Fig. 3d, the peaks for As(III), MMA, DMA, and AB became much smaller than those in the 21-h urine sample, and one noticeable new peak appeared at 410 s, which corresponded to unknown species 3 in the extracts from *hijiki*, as is seen in Fig. 2.

Urinary Excretion Patterns of Arsenic Metabolites after Ingestion of *Hijiki*. The observed concentrations of arsenic metabolites were calculated by comparing the peak areas with those of standard arsenic solutions. The observed concentrations of urinary arsenic excreted for 3 days are summarized in Table 2. These observed concentrations of arsenicals were then normalized to that of Na (ca. 3000 μg mL^{-1}) in each urine sample in order to compensate for the differences in water content. The Na-normalized concentrations of arsenicals in each urine sample are also summarized in Table 2, and were plotted as the urinary excretion patterns, as shown in Fig. 4.

As can be seen in Fig. 4, inorganic As(V) and As(III) were excreted rapidly 3 h after ingestion. The excretion of As(V) was at a maximum after 6 h, and it was not excreted after 18.5 h. As(III) provided the highest concentration in the 15-h urine sample. It can also be seen from Fig. 4 that the urinary excretion of methylated arsenic metabolites was much slower than that of inorganic ones. The concentrations of MMA, DMA, and AB gradually began to increase 10 h after ingestion and their highest concentrations were observed in the 21-h urine sample. These urinary arsenic excretion patterns were quite similar to those obtained by Yamauchi et al.,[14] where the water extract from *hijiki* was ingested. These results indicate that some part of As(V) taken from *hijiki* may be excreted rapidly in urine with no change of chemical form, and others may be reduced to As(III). Subsequently, some part of the reduced As(III) may be methylated to MMA and DMA in the body and excreted in urine. DMA showed higher values in most urine samples than MMA and AB. This result suggests that DMA is the end product of arsenic methylation in the human body. It can also be seen in Fig. 4 that DMA provided the excretion peaks in urine every 20 h (20, 40, and 60 h after ingestion). The first peak at 20 h may be due to arsenic methyl-

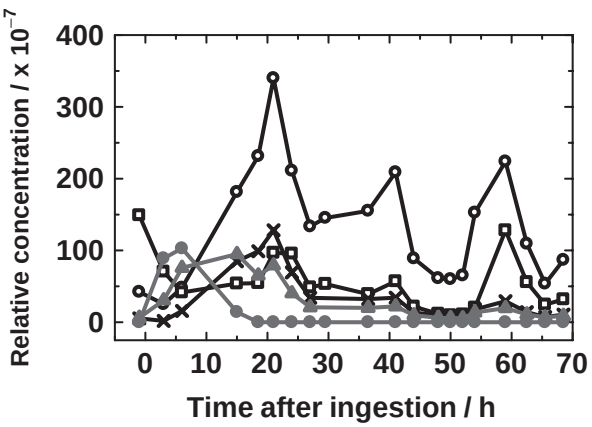

Fig. 4. Urinary excretion patterns of the relative concentrations of arsenic metabolites in human urine, normalized to the concentration of Na, as a function of the time after ingestion of *hijiki*. ●: As(V), ▲: As(III), ×: MMA, ○: DMA, □: AB.

ation, while the following two peaks of DMA excreted at 40 and 60 h after ingestion may be a metabolite due to the degradation of some arsenosugars, which are another major arsenic species in *hijiki*. Arsenosugars in the urine were preliminarily detected by ESI (electrospray ionization)-MS, although a detailed description is not given here.

Conclusion

It may be concluded that the ingestion of *hijiki* is not appreciably harmful to the human body, although a large amount of inorganic arsenics, As(V) and As(III), are contained in it. This is because such toxic arsenicals are rapidly excreted in urine or converted to non-toxic methylated compounds in the human body. In the previous paper,[10] the present authors reported that inorganic arsenicals were more abundantly distributed in the membrane of salmon egg cells, while organic arsenicals were mostly distributed in the cytoplasm of salmon egg cells. These results suggest that the methylation of inorganic arsenicals to organic ones may occur in cell membranes. Thus, methylation of As(V) and As(III) in *hijiki* may occur on a cell basis in some organs of the human body, which results in the detoxification of inorganic arsenicals. However, methylated arsenic species found in the present experiment were pentavalent ones. It has been pointed out that trivalent methylated arsenicals, which are arsenic methylation intermediates, are more toxic than inorganic arsenic species, and that methylation of arsenic may not be the entire detoxification process in humans.[19,20] Thus, further research is now in progress in order to elucidate the metabolic mechanisms of arsenic species including trivalent methylated ones in the human body.

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